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Regulated activity of PP2A-B55δ is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three reviewers, whose comments are attached below. I hope you understand if I do not go through each individual comment in this letter, but as you will see the referees express significant interest in the study and recommend publication after a suitable amount of revision. Referee #1 recommends publication after minor revision while both referee #2 and #3 find that although describing the mechanism of cell cycle regulation of PP2A-B55delta is beyond the scope of the current study some further molecular insight would beneficial and desirable. They suggest a number of different directions by which the study could be extended and seeming good experiments to address how this may be achieved. It is not necessary that all these points are addressed but some further insight should be provided. Should you be able to address the raised concerns we would be willing to consider a revised version of your manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your revisions included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Review of EMBOJ-2009-70479 (Hunt)

In this paper, Mochida and colleagues have investigated the identity of the phosphatase(s) that dephosphorylates Cdk substrates and therefore antagonizes the entry into mitosis and promotes the subsequent exit from mitosis. To address this question, they have prepared or obtained antibodies to most of the catalytic phosphatase subunits, depleted them from egg extracts, and examined remaining phosphatase activity in the extracts. Having identified PP2A as the candidate through this approach, the authors continued by removing many of the regulatory PP2A B subunits from the extracts. These experiments allowed the authors to focus on the holoenzyme containing the B55δ subunit as the relevant enzyme. By depleting this enzyme, the authors provide convincing evidence that it controls dephosphorylation of mitotic substrates. Depleted extracts enter mitosis more rapidly. Conversely, extracts contain extra holoenzyme go into mitosis more slowly or barely at all, depending on the final concentration of this enzyme.

Overall, I believe that this paper describes a substantial amount of work with convincing data that reveals a significant point. It is natural to wonder how this enzyme is regulated, but that is probably too much to ask for in an initial report. I would recommend publication pending some minor revisions.

Points

1. Figure 1 (B and C) and Figure 4 (D). It would be easier for the reader if the authors labeled the graphs directly.

2. Figure 3A. It is an exaggeration to say that p55δ is removed. It looks more like 70%, which corresponds to the reduction in phosphatase activity.

3. Discussion (page 15, line 16). Should be "dephosphorylation of Cdc25"? In general, the Discussion could have been a little clearer.

4. The authors use both Fzy and Cdc20 to refer to phosphatase substrate in text.

5. The grammar is rough in some places (for example, incomplete sentence at the end of first paragraph of introduction).

Referee #2 (Remarks to the Author):

This paper documents the identification of a phosphatase activity that helps control progression through mitosis in frog embryonic extracts. It is well established that dephosphorylation of Cdk substrates is critical for exit from mitosis, but there is considerable confusion and interspecies variation in the phosphatases that are involved. The present paper clarifies the situation nicely by showing, quite convincingly, that the major anti-Cdk phosphatase in late mitotic frog extracts is PP2A, together with its regulatory subunit B55δ. Immunodepletion experiments reveal that PP2A and B55d comprise the predominant phosphatase in the extract, and a nice immunodepletion experiment in Figure 4C shows clearly that removal of PP2A-B55d from cycling extracts blocks the dephosphorylation of Cdk substrates in late mitosis. Figure 5 shows that the addition of extra phosphatase delays entry into mitosis, also consistent with the notion that this phosphatase opposes Cdk activity.

The experiments are well done, the data are clear, and I am quite convinced that PP2A-B55d is a key phosphatase in late mitotic control in this system. This is an important result and worthy of publication. However, I also feel that the paper would be more compelling if one or two issues were pursued in more depth. The authors might want to consider the following points:

1. As the authors point out early in the paper, the anti-Cdk phosphatase activity oscillates in cell cycle extracts. After identifying PP2A as the phosphatase, they do not go back to confirm that it is indeed PP2A activity that oscillates in the cell cycle. This issue could be addressed by developing a method to measure phosphatase activity in anti-PP2A immunoprecipitates, so that they could show directly that PP2A-associated activity in immunoprecipitates oscillates in the cell cycle. If they had this assay, they could then make great strides toward understanding the mechanisms underlying PP2A regulation (next point).

2. Regulation of PP2A-B55d. The authors point out in their discussion that the big remaining question is how the phosphatase is turned on in late mitosis. Their evidence clearly points to the idea that Cdk activity inhibits PP2A-B55d, and that some other phosphatase must therefore activate PP2A-B55d in late mitosis. As the authors point out, an obvious candidate for this regulatory phosphatase is PP1. This would be consistent with the immunodepletion results in Figure 2, which show that depletion of PP2A removes almost all activity and yet depletion of PP1 also removes a significant amount. Considering that the authors possess an anti-PP1 antibody that depletes effectively, would it not be possible to test the effects of PP1 depletion on the activation of PP2A-B55d that results from addition of p27 to mitotic extracts? This would best be done if the authors had a PP2A immunoprecipitate assay as described in point 1 above.

The authors imply that regulation of PP2A is post-translational, and their experiments are certainly consistent with this. However, this point would be driven home more effectively if they simply did a western blot of PP2A and B55d across the cell cycle, to confirm that levels of the two subunits do not change. This experiment might also unveil a phosphate-dependent mobility shift that could provide clues about mechanisms of regulation.

3. The authors point out the puzzling result that the APC/C seems to be shut off after mitosis in the PP2A-depleted extract, even though Apc3 remains phosphorylated. One possibility is that Cdc20 is ubiquitinated by active APC/C (as in the spindle checkpoint, as suggested recently by Pines), resulting in its destruction. Are anti-Cdc20 antibodies available for checking the levels of Cdc20 in these extracts?

Minor technical points:

-when discussing Figure 5, the authors mention that cyclin B did not reappear in extracts treated with 1.5X phosphatase. Is this meaningful, given that cyclin B also did not reappear in the mock-treated extract at the top of the figure? Isn't the mock extract at the top of Figure 5 equivalent to the mock-treated extract at the top of Figure 4C? Why do they behave differently? - it is not clear from the figure legends if there is some difference in the methods here.

-Figure 6 seems unnecessary and could be removed.

Referee #3 (Remarks to the Author):

The manuscript "Regulated activity of PP2A-B55δ is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts" addresses the important question of how M phase phosphoproteins are dephosphorylated at exit from mitosis. Based on immunodepletion and addition experiments in Xenopus egg extracts, the authors conclude that PP2A-B55δ is the major phosphatase controlling M phase substrate dephosphorylation. Although their data are certainly consistent with an important role for this complex, some additional experimentation would make this a stronger manuscript, more suitable for publication in EMBOJ. I also believe that some characterization of mechanisms regulating the complex would be appropriate, even if it falls short of a complete understanding.

1) Although the depletion data implicating PP2A-B55δ in M phase substrate dephosphorylation are compelling and the fact that phosphatase activity directed against M phase phosphoproteins is convincing, the link between the two is missing. Assaying phosphatase activity in PP2A immunoprecipitates at different cell cycle stages rather than assaying the total extract (Fig1B and

1C) would provide better direct evidence for cell cycle regulation of PP2A- B55δ .

2) Although a full characterization of PP2A- B55δ cell cycle regulation is beyond the scope of this manuscript, a basic characterization that would determine 1) whether B55-M phase substrate association is cell cycle regulated or 2) B55-PP2A association is cell cycle regulated (or both) would be appropriate.

3) While the in vitro Fry-S50 dephosphorylation data support the idea that PP2A- B55δ directly dephosphorylates mitotic substrates, peptide dephosphorylation data can be highly misleading. The story would be more convincing if more physiological substrates were examined for direct dephosphorylation (eg: immunoprecipitates of APC3, Cdc20 and/or Cdc25)

4) In the experiment shown in Fig4C, CDK kinase activity reappeared rapidly after the first M exit. Performing the same depletion experiment in an "M extract plus p27" setting would provide stronger evidence for the requirement of B55δ as it would eliminate the complication of the APC effect.

5) Do the authors have any evidence that B55δ -PP2A also regulates M phase substrate dephosphorylation in mammalian cells?

1st Revision - authors' response 17 June 2009

Referee #1 :

In this paper, Mochida and colleagues have investigated the identity of the phosphatase(s) that dephosphorylates Cdk substrates and therefore antagonizes the entry into mitosis and promotes the subsequent exit from mitosis. To address this question, they have prepared or obtained antibodies to most of the catalytic phosphatase subunits, depleted them from egg extracts, and examined remaining phosphatase activity in the extracts. Having identified PP2A as the candidate through this approach, the authors continued by removing many of the regulatory PP2A B subunits from the extracts. These experiments allowed the authors to focus on the holoenzyme containing the B55δ subunit as the relevant enzyme. By depleting this enzyme, the authors provide convincing evidence that it controls dephosphorylation of mitotic substrates. Depleted extracts enter mitosis more rapidly. Conversely, extracts contain extra holoenzyme go into mitosis more slowly or barely at all, depending on the final concentration of this enzyme.

Overall, I believe that this paper describes a substantial amount of work with convincing data that reveals a significant point. It is natural to wonder how this enzyme is regulated, but that is probably too much to ask for in an initial report. I would recommend publication pending some minor revisions.

1. Figure 1 (B and C) and Figure 4 (D). It would be easier for the reader if the authors labeled the graphs directly.

> *We have added labels to these figures.*

2. Figure 3A. It is an exaggeration to say that p55δ is removed. It looks more like 70%, which corresponds to the reduction in phosphatase activity.

> *This is correct, and we have amended the description.*

3. Discussion (page 15, line 16). Should be "dephosphorylation of Cdc25"? In general, the Discussion could have been a little clearer.

> *Right. We changed "phosphorylation" to dephosphorylation. We hope the Discussion is generally improved.*

4. The authors use both Fzy and Cdc20 to refer to phosphatase substrate in text.

> *We now use Fizzy or Fizzy/Cdc20.*

5. The grammar is rough in some places (for example, incomplete sentence at the end of first paragraph of introduction).

> *We have gone carefully through the paper trying to say exactly what we mean, as clearly as we can. We made a number of minor changes intended to improve the sense and flow.*

Referee #2 (Remarks to the Author):

This paper documents the identification of a phosphatase activity that helps control progression through mitosis in frog embryonic extracts. It is well established that dephosphorylation of Cdk substrates is critical for exit from mitosis, but there is considerable confusion and interspecies variation in the phosphatases that are involved. The present paper clarifies the situation nicely by showing, quite convincingly, that the major anti-Cdk phosphatase in late mitotic frog extracts is PP2A, together with its regulatory subunit B55δ. Immunodepletion experiments reveal that PP2A and B55δ comprise the predominant phosphatase in the extract, and a nice immunodepletion experiment in Figure 4C shows clearly that removal of PP2A-B55δ from cycling extracts blocks the dephosphorylation of Cdk substrates in late mitosis. Figure 5 shows that the addition of extra phosphatase delays entry into mitosis, also consistent with the notion that this phosphatase opposes Cdk activity.

The experiments are well done, the data are clear, and I am quite convinced that PP2A-B55δ is a key phosphatase in late mitotic control in this system. This is an important result and worthy of publication. However, I also feel that the paper would be more compelling if one or two issues were pursued in more depth. The authors might want to consider the following points:

1. As the authors point out early in the paper, the anti-Cdk phosphatase activity oscillates in cell cycle extracts. After identifying PP2A as the phosphatase, they do not go back to confirm that it is indeed PP2A activity that oscillates in the cell cycle. This issue could be addressed by developing a method to measure phosphatase activity in anti-PP2A immunoprecipitates, so that they could show directly that PP2A-associated activity in immunoprecipitates oscillates in the cell cycle. If they had this assay, they could then make great strides toward understanding the mechanisms underlying PP2A regulation (next point).

> *We have tested this, but we do not see any difference in PPase activity between immunoprecipitated fractions from either interphase or M phase. This is probably because of, we presume, either some auto-activation (auto-dephosphorylation) or the dissociation of inhibitory factor(s). This is also suggested from the extract dilution experiment (shown in the supplementary figures). When the extract is diluted, the difference of phosphatase activity between interphase and M-phase is lost. Probably, the inhibitor is not very tight-binding.*

2. Regulation of PP2A- B55δ. The authors point out in their discussion that the big remaining question is how the phosphatase is turned on in late mitosis. Their evidence clearly points to the idea that Cdk activity inhibits PP2A- B55δ, and that some other phosphatase must therefore activate PP2A- B55δ in late mitosis. As the authors point out, an obvious candidate for this regulatory phosphatase is PP1. This would be consistent with the immunodepletion results in Figure 2, which show that depletion of PP2A removes almost all activity and yet depletion of PP1 also removes a significant amount. Considering that the authors possess an anti-PP1 antibody that depletes effectively, would it not be possible to test the effects of PP1 depletion on the activation of PP2A-B55δ that results from addition of p27 to mitotic extracts? This would best be done if the authors had a PP2A immunoprecipitate assay as described in point 1 above.

> *As described above, once PP2A-B55δ is purified by immunoprecipitation, the difference of its activity disappears.*

The authors imply that regulation of PP2A is post-translational, and their experiments are certainly consistent with this. However, this point would be driven home more effectively if they simply did a western blot of PP2A and B55δ across the cell cycle, to confirm that levels of the two subunits do not change. This experiment might also unveil a phosphate-dependent mobility shift that could provide clues about mechanisms of regulation.

> *No change in amount or mobility was observed in B55δ or PP2Ac on SDS-PAGE, as shown in the supplementary figure 2.*

3. The authors point out the puzzling result that the APC/C seems to be shut off after mitosis in the PP2A-depleted extract, even though Apc3 remains phosphorylated. One possibility is that Cdc20 is ubiquitinated by active APC/C (as in the spindle checkpoint, as suggested recently by Pines), resulting in its destruction. Are anti-Cdc20 antibodies available for checking the levels of Cdc20 in these extracts?

> *Since there is no Fzr protein in Xenopus egg extracts (Lorca et. al., (1998) EMBO J., 17:3565-3575), which is required for Fzy degradation, it is unlikely that Fzy levels oscillate in these extracts. We did, however, check this point and found no significant changes in the amount of Fzy during the cell cycles in these egg extracts (See Supplementary Figure 2).*

Minor technical points:

-when discussing Figure 5, the authors mention that cyclin B did not reappear in extracts treated with 1.5X phosphatase. Is this meaningful, given that cyclin B also did not reappear in the mock-treated extract at the top of the figure? Isn't the mock extract at the top of Figure 5 equivalent to the mock-treated extract at the top of Figure 4C? Why do they behave differently? - it is not clear from the figure legends if there is some difference in the methods here.

> *We have removed this discussion about the APCís activity, which we do not really understand, and as the reviewer points out, looks rather variable. The activities of different extracts are just different, another thing thatís not well-understood.*

-Figure 6 seems unnecessary and could be removed.

> *We agree, and have removed this figure.*

Referee #3:

The manuscript "Regulated activity of PP2A- B55δ is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts" addresses the important question of how M phase phosphoproteins are dephosphorylated at exit from mitosis. Based on immunodepletion and addition experiments in Xenopus egg extracts, the authors conclude that PP2A- B55δ is the major phosphatase controlling M phase substrate dephosphorylation. Although their data are certainly consistent with an important role for this complex, some additional experimentation would make this a stronger manuscript, more suitable for publication in EMBOJ. I also believe that some characterization of mechanisms regulating the complex would be appropriate, even if it falls short of a complete understanding.

1) Although the depletion data implicating PP2A- B55δ in M phase substrate dephosphorylation are compelling and the fact that phosphatase activity directed against M phase phosphoproteins is convincing, the link between the two is missing. Assaying phosphatase activity in PP2A immunoprecipitates at different cell cycle stages rather than assaying the total extract (Fig1B and 1C) would provide better direct evidence for cell cycle regulation of PP2A-B55 .

> *As described above, once immunoprecipitated, PP2A- B55δ does not show any cell-cycle regulated activity. So this is impossible to do so far. Either the phosphatase activates itself during the procedure, or we lose a loose-binding inhibitor (or a co-factor required for the supposed inhibitor to work).*

2) Although a full characterization of PP2A- B55δ cell cycle regulation is beyond the scope of this manuscript, a basic characterization that would determine 1) whether B55-M phase substrate association is cell cycle regulated or 2) B55-PP2A association is cell cycle regulated (or both) would be appropriate.

> We did not find any binding between PP2A- B55δ and its substrates. The bindings of B55δ and PP2A catalytic subunits are same both in interphase & M phase (please see the supplementary figure 2).

3) While the in vitro Fry-S50 dephosphorylation data support the idea that PP2A- B55δ directly dephosphorylates mitotic substrates, peptide dephosphorylation data can be highly misleading. The story would be more convincing if more physiological substrates were examined for direct dephosphorylation (eg: immunoprecipitates of APC3, Cdc20 and/or Cdc25)

> It is best if we can detect the speed of dephosphorylation of one particular phosphorylation site of a particular protein. But if you use multiply-phsophorylated substrate in phosphatase assay, the activity you get is a mixture of phosphatase activity against different phosphorylation sites. We actually observed that if we use histone H1 phosphorylated by CDK at more than 10 S/T residues, the phosphatase activity changes very little through the cell-cycle. We checked Op18/Stathmin, which has only one CDK site, as a substrate for phosphatase assays. But we found that recombinant Op18 was a rather poor substrate for CDK phosphorylation in vitro and also a poor substrate for phosphatase, making it difficult to evaluate the data. We would very much like to have a more natural substrate to test.

4) In the experiment shown in Fig 4C, CDK kinase activity reappeared rapidly after the first M exit. Performing the same depletion experiment in an "M extract plus p27" setting would provide stronger evidence for the requirement of B55δ; as it would eliminate the complication of the APC effect.

This is a very important point, and following it up has changed our interpretation of the function of B55δ. As shown in the newly added figure 6, B55δ was not essential for the dephosphorylation in the p27 experiment (when B55δ was depleted from a mitotic extract), but is required when B55δ is removed during the preceding interphase & cyclin is removed by a physiological degradation pathway (Figure 4) or by using an antibody affinity resin (Figure 6A and B). It is possible that normally, Wee1 plays some role in inhibiting the last traces of CDK1 activity and when B55δ is absent, Wee1 cannot be dephosphorylated and reactivated, and so traces of residual cyclin B/CDK1 activity remain to keep the extracts in M-phase.

5) Do the authors have any evidence that B55δ -PP2A also regulates M phase substrate dephosphorylation in mammalian cells?

> We do not know anything so far. It would be quite difficult to do.

2nd Editorial Decision 01 July 2009

Your manuscript has been reviewed once more by the original referees. Overall the referees find that the manuscript contains data of interest to the field and support publication in the EMBO Journal. I would like to point out that in light of the results of the mitotic depletion experiments referee #3 requests that the title and abstract are modified and would like clarification of a number of issues in the text.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. Given the recent data on PP1, I would grateful if these changes could be made a soon as possible so we can quickly proceed with publication.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript is less satisfying with the revised model and in view of recent data from Kornbluth lab on PP1. However, the work is well performed and does provide a solid piece to the puzzle of cell cycle regulation by phosphatases.

Referee #2 (Remarks to the Author):

The authors have responded effectively to all of my previous concerns. It is clear from their rebuttal that studies of PP2A-B55d regulation will require the development of technical approaches that are beyond the scope of this paper. As before, this paper represents an important advance in our understanding of mitotic regulation, and I would recommend publication without delay.

Referee #3 (Remarks to the Author):

The revised version of this manuscript now suggests that PP2A- B55δ is unlikely to be the primary phosphatase regulating dephosphorylation of Cdk substrates at mitotic exit and, although the data are clean, their interpretation is not entirely clear. At the very least, the authors should tone down their conclusions. The title, abstract, and end of the introduction should be amended. In particular: 1) The title would more accurately be something like: Regulated activity of PP2A- B55δ is crucial for controlling entry mitosis and influences mitotic exit in Xenopus egg extracts 2) In the abstract, the authors say: The activity of this form of PP2A is regulated during cell cycle - high in interphase and suppressed during mitosis. Without the requested immunoprecipitation experiments, this conclusion needs to be softened. 3) At the end of the introduction, the authors say: "Depletion of this form of PP2A strongly advances entry into mitosis in Xenopus egg extracts, and the depleted extracts are unable to exit mitosis despite having degraded cyclin B and turned off Cdk activity." Yet in the response to reviewers, the authors say " It is possible that normally, Wee1 plays some role in inhibiting the last traces of CDK1 activity and when B55δ is absent, Wee1 cannot be dephosphorylated and reactivated, and so traces of residual cyclin B/CDK1 activity remain to keep the extracts in M-phase". These can't both be true-ie if depletion of PP2A-B55 causes inhibition of mitotic exit because of residual Cyclin B/Cdk activity, then the extracts are not stuck in mitosis despite having degraded Cyclin B and turned off Cdk activity.
In the end, the reader is left a bit dissatisfied. Is it Wee1 that is the target of B55 that results, ultimately, in effects on exit from mitosis when B55 is depleted during the previoius interphase? Is this a physiologically relevant form of regulation or is the primary effect of B55δ on entry into, rather than exit from, mitosis?

2nd Revision - authors' response 10 July 2009

Here is the revised revised paper about mitotic phosphatases. Thank you for getting it reviewed again so quickly.

We have tried to meet the specific points raised by reviewer number 3 as far as possible.

1. We disagree about the title, and think the original title is still perfectly accurate, and not misleading.

2. We modified the abstract by saying that the activity against our model substrate is regulated, rather than that the activity of PP2A-B55 is regulated. This is more accurate, and true.

3. As far as exit from mitosis goes, we now place more emphasis on Wee1, its regulation and possible role at the end of mitosis in the light of a recent paper from Gary Gorbsky, (this in the

Introduction) and further de-emphasize the idea that PP2A-B55 is the *only* anti-mitotic phosphatase in the Discussion.

We understand the dissatisfaction felt by Reviewer numbers 1 and 3; we feel it ourselves! But only further experiments will unravel the truth. These may take some time, and require fresh reagents.

We think the paper has been substantially improved, thanks to the reviewers' comments. They saved us from quite serious over-interpretation of the data.